p-ISSN : 2788-4961 | e-ISSN : 2788-418X
DOI(Journal): 10.31703/giddr
DOI(Volume): 10.31703/giidr/.2024(IX)
DOI(Issue): 10.31703/giidr.2024(IX.I)

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GLOBAL IMMUNOLOGICAL & INFECTIOUS DISEASES REVIEW

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VOL. IX, ISSUE I, WINTER (MARCH-2024)

Double-blind Peer-review Research Journal
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Humanity Publications(HumaPub)
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Doi:https://dx.doi.org/10.31703

Article Title

Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease Virus

Global Immunological &Infictious Diseases Review
p-ISSN: 2788-4961 **e-ISSN:** 2788-418X
DOI(journal): 10.31703/giidr
Volume: IX (2024)
DOI (volume): 10.31703/giidr.2024(IX)
Issue: (Winter-March 2024)
DOI(Issue): 10.31703/giidr.2024(IX-I)
Home Page
www.giidrjournal.com
Volume: IX (2024)
https://www.giidrjournal.com/Current-issues
Issue: I-Winter (March-2024)
https://www.giidrjournal.com/Current-issues/9/1/2024
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Abstract

Foot and Mouth Disease (FMD) is a highly contagious viral illness that predominantly affects cloven-hoofed animals. To identify and profile the predominant FMDV serotypes circulating in Baluchistan bovine and buffalo populations. Twenty tissue samples were collected and analyzed using sandwich indirect ELISA and RT-PCR to identify the presence and genetic characteristics of FMD virus. We identified 'O' serotype of FMD virus in all specimens. RT-PCR analysis yielded an impressive 90% positivity rate, confirming its diagnostic efficacy. While observations of LFBK cell line lead to the isolation of 18 distinct viruses with distinct Cytopathic Effects, two samples lacked detectable CPEs. Lower CT values, those observed in sample PK-16 with CT of 15, indicated higher initial concentration of the target nucleic acid, indicating a potential for higher viral burden. The endemic prevalence of 'O' serotype in Balochistan necessitated targeted serotype-specific interventions, strengthened surveillance and strategic vaccination campaigns.

Keywords: Characterization; Disease Surveillance; Sandwich ELISA; RT-PCR; Serotypes.

Authors:

Mahnoor Mazhar: (Coprrespondant author)
Research Assistant, Department of Biosciences , COMSAT University, Islamabad, Pakistan.
(Email: mahnoormazhar2@gmail.com)
Rida e Tahreem Akram: PhD Scholar, National Centre for Bioinformatics, Quaid I Azam Universitry, Islamabad, Pakistan.
Maryam Hayat: Assistant Scientific Officer, Pakistan Agricultural Research Institute, Arid Zone Research Institute, Bahawalpur, Punjab, Pakistan.
Abou Bakar Siddique: Scientific Officer, Pakistan Agricultural Research Institute, Arid Zone Research Institute, Bahawalpur, Punjab, Pakistan.
Hasooba Hira: PhD Scholar, PhD Scholar, Department of Zoology, University of Agriculture, Faisalabad. Punjab, Pakistan.
Shakeela Naheed: PhD Scholar, Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Punjab, Pakistan.`

Pages: 1-9
DOI: 10.31703/giidr.2024(IX-I).01
DOI link: https://dx.doi.org/10.31703/giidrr.2024(IX-I).01
Article link: http://www.giidrrjournal.com/article/A-b-c
Full-text Link: https://giidrrjournal.com/fulltext/
Pdf link: https://www.giidrjournal.com/jadmin/Auther/31rvlolA2.pdf

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Doi:https://dx.doi.org/10.31703

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Citing this Article

01		**Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease**						
		Author	Mahnoor Mazhar Rida e Tahreem Akram Maryam Hayat Abou Bakar Siddique Hasooba Hira Shakeela Naheed		**DOI**	10.31703/giidr.2024(IX-I).01		
Pages		1-9	**Year**	2024	**Volume**	IX	**Issue**	I
Referencing & Citing Styles	**APA**	Mazhar, M., Akram, R. e. T., Hayat, M., Siddique, A. B., Hira, H., & Naheed, S. (2024). Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease. *Global Immunological &Infictious Diseases Review*, *IX*(I), 1-9. https://doi.org/10.31703/giidr.2024(IX-I).01						
	CHICAGO	Mazhar, Mahnoor, Rida e Tahreem Akram, Maryam Hayat, Abou Bakar Siddique, Hasooba Hira, and Shakeela Naheed. 2024. "Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease." *Global Immunological &Infictious Diseases Review* IX (I):1-9. doi: 10.31703/giidr.2024(IX-I).01.						
	HARVARD	MAZHAR, M., AKRAM, R. E. T., HAYAT, M., SIDDIQUE, A. B., HIRA, H. & NAHEED, S. 2024. Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease. *Global Immunological &Infictious Diseases Review* IX**,** 1-9.						
	MHRA	Mazhar, Mahnoor, Rida e Tahreem Akram, Maryam Hayat, Abou Bakar Siddique, Hasooba Hira, and Shakeela Naheed. 2024. 'Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease', *Global Immunological &Infictious Diseases Review*, IX: 1-9.						
	MLA	Mazhar, Mahnoor, et al. "Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease." *Global Immunological &Infictious Diseases Review* IX.I (2024): 1-9. Print.						
	OXFORD	Mazhar, Mahnoor, et al. (2024), 'Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease', *Global Immunological &Infictious Diseases Review* IX (I), 1-9.						
	TURABIAN	Mazhar, Mahnoor, Rida e Tahreem Akram, Maryam Hayat, Abou Bakar Siddique, Hasooba Hira, and Shakeela Naheed. "Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease." *Global Immunological &Infictious Diseases Review* IX, no. I (2024): 1-9. https://dx.doi.org/10.31703/giidr.2024(IX-I).01.						

e-ISSN: 2788-418X **Volume:** IX (2024) **Issue:** I-Winter (March-2024) **p-ISSN:** 2788-4961

Global Immunological &Infictious Diseases Review
www.giidrjournal.com
DOI:http://dx.doi.org/10.31703/glsr

Pages: 1-9 **URL:**https://doi.org/10.31703/giidr.2024(IX-I).01 **Doi:** 10.31703/giidr.2024(IX-I).01

Cite Us

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Isolation, Genetic and Antigenic Characterization of Circulating Foot and Mouth Disease Virus

Abstract

Foot and Mouth Disease (FMD) is a highly contagious viral illness that predominantly affects cloven-hoofed animals. To identify and profile the predominant FMDV serotypes circulating in Baluchistan bovine and buffalo populations. Twenty tissue samples were collected and analyzed using sandwich indirect ELISA and RT-PCR to identify the presence and genetic characteristics of FMD virus. We identified 'O' serotype of FMD virus in all specimens. RT-PCR analysis yielded an impressive 90% positivity rate, confirming its diagnostic efficacy. While observations of LFBK cell line lead to the isolation of 18 distinct viruses with distinct Cytopathic Effects, two samples lacked detectable CPEs. Lower CT values, those observed in sample PK-16 with CT of 15, indicated higher initial concentration of the target nucleic acid, indicating a potential for higher viral burden. The endemic prevalence of 'O' serotype in Balochistan necessitated targeted serotype-specific interventions, strengthened surveillance and strategic vaccination campaigns.

Keywords: Characterization; Disease Surveillance; Sandwich ELISA; RT-PCR; Serotypes

Authors:

Mahnoor Mazhar: (Coprrespondant author)
Research Assistant, Department of Biosciences , COMSAT University, Islamabad, Pakistan.
(Email: mahnoormazhar2@gmail.com)

Rida e Tahreem Akram: PhD Scholar, National Centre for Bioinformatics, Quaid I Azam Universitry, Islamabad, Pakistan.

Maryam Hayat: Assistant Scientific Officer, Pakistan Agricultural Research Institute, Arid Zone Research Institute, Bahawalpur, Punjab, Pakistan.

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Hasooba Hira: PhD Scholar, PhD Scholar, Department of Zoology, University of Agriculture, Faisalabad. Punjab, Pakistan.

Shakeela Naheed: PhD Scholar, Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Punjab, Pakistan.`

Contents

Introduction

Foot-and-mouth disease documented since Fracastorius's writings in 1514 Italy, remains a formidable adversary to global livestock industries (Jamal &Belsham, 2013b). The causative agent (FMDV) was subsequently identified as a filterable infectious organism (Li et al., 2021). It belongs to the Aphthovirus genus of Picornaviridae, mostly infecting the cloven-footed animals. It exhibits a wide range of variation, having seven serotypes: A, O, C, Asian 1, and SAT 1-3. The rapid spread of the virus, either through airborne particles or close physical touch, makes it even more difficult to control (Knowles& Samuel, 2003)

The FMD outbreak has had a significant and far-reaching impact on the country's economy. While adult cattle may have a relatively low death rate, there are negative consequences such as reduced milk production, fertility problems, and declined ability to be used for draught purposes. In Pakistan, where large animal populations including the Asian buffalo, the

consequences of the disease are clearly evident (Ferrari et al., 2013) (Ansari-Lari et al., 2017).

Historical sources depict the complex portrayal of FMDV. Genetic investigations have provided evidence of the genome of FMDV which is a single-stranded positive-sense RNA molecule of approximately 8.5 kb (Pierce et al., 2023). This RNA molecule encodes a single polyprotein. Following proteolytic cleavage, this polyprotein generates four structural proteins and ten non-structural proteins. The VP1 protein in this array is particularly notable due to its significant diversity, making it a key element of the genetic landscape and epidemiology of FMDV (Sarry et al., 2022).

Pakistan, despite dealing with the persistent existence of FMD, observes an inconsistent approach to its care. The unpredictable path of FMDV can be attributed in part to the lack of thorough genetic research on the virus, especially in relation to Asian buffaloes (Naqvi et al., 2022) (Farooq et al., 2018b) Considering FMDV's worldwide classification into 07 different strains, it is crucial to continuously monitor and genetically characterize these strains. In Pakistan, serotype O is the dominant strain of the Euro-Asia viral reservoir (Pool 3) and it shares genetic similarities with viruses from neighboring regions (Jamal et al., 2011)

Pakistan's blueprint for FMD mitigation traditionally leans on vaccination. However, this strategy encounters hurdles such as unregulated livestock mobility, archaic vaccines, and FMDV strains' nimble mutation capabilities. The narrative becomes even more intricate when accounting for carrier animals, and potential Trojan horses, courtesy of FMDV's ability to nestle within hosts, facilitating covert disease transmission(Farooq et al., 2018)(Yadav et al., 2022).

Given FMD's economic importance and its multifaceted epidemiological profile, which is accentuated by its genetic dynamism, our objective was to identify and meticulously profile the predominant FMDV serotypes in Balochistan's cattle and buffalo population. We charted a course to comprehend FMDV's persistence in buffaloes and employed molecular techniques to investigate the genetic blueprints of the circulating strains.

Material and Methods

Collecting Tissue Samples

Over the course of seven months, from February to September 2022, twenty tissue samples were thoroughly examined in Balochistan province. Tongue and buccal mucosa were the primary harvesting locations for epithelial tissue, but occasionally samples were also taken from limbs. A special medium containing antibiotics including penicillin, neomycin sulfate, polymyxin B sulfate, and Mycostatin was used to preserve the integrity of the epithelial samples during transport. This medium's pH was kept between 7.2 and 7.7 with the help of glycerol and phosphate buffer. For accurate record-keeping, each sample was accompanied by a detailed Performa that included sections on disease etiology, vaccine types used, husbandry practices, observed clinical symptoms, mortality, and morbidity. One epidemiological entity was designated for each individual epidemic. Following collection, samples were transported to Islamabad's National Agricultural Research Center (NARC) in a refrigerated vehicle and were frozen at -80 degrees Celsius.

Tissue Sample Preparation

Prior to tissue processing, approximately 1g of epithelial tissue from the primary sample was washed with PBS. This tissue was then finely pulverized in a 1 cc PBS solution using a sterile pestle and mortar. For ELISA, undiluted, clarified supernatant fluid from cell culture or 10% original sample solution was utilized. The culture's supernatant was subsequently collected for antigen analysis after centrifugation.

Sandwich Indirect ELISA for Detection of FMDV Antigens

For the identification of FMDV serotypes, an indirect sandwich ELISA reagent was used. Using specific monoclonal antibodies against FMD virus serotypes, antigenic ELISA was performed to determine the serotype of the isolated strains. The micro-titration plate was labeled. Various FMDV-serotype-specific rabbit antisera were used to classify the various sections on multiwell plates. The detailed procedure included coating rows with rabbit antiserum at specific dilutions and adhering to the detailed incubation and rinsing protocol. This was followed by the addition of attenuated guinea pig antiserum and then an incubation period. The micro-titration plate's wells were filled with chromogen substrate and incubated once more. The procedure concluded with the addition of a stop solution and ELISA reader analysis.(Mallick et al., 2023).

Isolation and Transmission of FMDV

The low passage fetal bovine kidney cell line (LFBK) maintained in the virology division of NARC, Islamabad, was utilized to extract FMDV isolates.

LFBK Cell Sub-Culturing

All LFBK-related procedures were performed in the flow cabinet. Using the split ratio technique, cells were sub-cultured. Until the monolayer was formed, a detailed protocol involving PBS, trypsin, Fetal bovine serum, and Dulbecco Modified Eagle Medium (DMEM) was followed (Mughal, 2017).

Infectious Agent in Cell Culture

The procedure commenced with the formulation of a fivefold dilution of homogenized epithelial sample supernatant. The flask containing the cells was examined for cytopathic effects (CPE) following multiple inoculation steps. If specific CPE was observed in a significant proportion of the cells, it was determined that FMDV had been isolated.

Establishment of FMDV Isolates

This required identifying the FMDV isolated from cell culture by utilizing multiple techniques, including specific CPE, indirect sandwich ELISA, and RT-PCR. After 3rd passage, the supernatant was extracted from any flask that displayed CPE.

RNA Extraction and Isolation Confirmation

The QIAamp® Viral RNA Mini reagent was used for this procedure. The entire extraction procedure involved a meticulous procedure in which the kit's efficacy was initially evaluated using positive controls. The research material then underwent an extraction procedure.

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

RNA was subjected to RT-PCR for detecting viral RNA after it was extracted. Standard protocol was followed to create the master mix and identify the viral RNA in the samples (Table 1) through established forward and reverse primers (Park et al., 2022) (Table 2).

Documentation on Gel

After electrophoresis, the gel was viewed with the gel documentation apparatus under UV light.

RT-PCR for Verification of FMDV Isolation

Using standard RT-PCR techniques and the acquired RNA, the isolated FMDV was then recognized. This step required the use of particular primers and probes and followed the standard protocol(Paixão et al., 2008).

Statistical Analysis

Using SPSS 25.0, data were analyzed. At p0.05, differences were deemed statistically significant.

Table 1

MastermixforRT-PCR

Reagent	Vol/reaction(µl)	TotalReactions	Total volume(µl)
2XRT-PCRbuffer1	12	10	120
25XRT-PCRenzymemix	1	10	10
Nucleasefreewater	7	10	70
Forwardprimer(50pmol)	1	10	10
ReversePrimer(50 pmol)	1	10	10

Table 2

Sequenceofforward, reverse primers and probe for RT-PCR

ForwardPrimer	5'ACTGGGTTTTACAAACCTGTGA3'
ReversePrimer	5'GCGAGTCCTGCCACGGA3'
Probe	6FAM5TCCTTTGCACGCCGTGGGAC-TAMRA

Results

The dataset provided an overview of samples collected from female bovines in Balochistan, Pakistan, for this study. Quetta is the primary collection region, with subregions being highlighted. The sampled animals were primarily cattle, with a few buffaloes, and their ages ranged from one to six years. Notably, every sample contained the 'O' serotype of the FMD virus. This serotype uniformity suggested that the 'O' variant

is prevalent among the bovine population in these regions of Balochistan (Table 3).

CPEs were identified in the LFBK cell line, which resulted in the isolation of 18 distinct viruses. However, two samples lacked any detectable CPEs. A new confluent LFBK cell line was established, with cells exhibiting complete adhesion and an elongated appearance. After the formation of a cohesive layer, the cell line was exposed to epithelial tissue samples derived from symptomatic organisms. The resulting CPEs were characterized by cellular contraction, darkening, and the formation of circular aggregates (Figure 1). The results of RT-PCR assay used to detect FMD virus in the collected samples. The integral Cycle Threshold (CT) values served as indicators of viral burden in each sample. Lower CT values, such as those observed in sample PK-16 with CT of 15, indicated a higher initial concentration of the target nucleic acid, indicating a potential for higher viral burden. In contrast, samples with elevated CT values, such as PK-07 and PK-11, suggested lower initial concentrations of the target sequence, thereby indicating a lowered viral burden. Some samples, including PK-01, PK-02, PK-03, and PK-08, contained dashes, which indicated the absence of detectable virus (Table 4).

In the course of our extensive investigation into the presence and genetic characteristics of FMD virus in cattle samples, RT-PCR revealed that 18 of the twenty samples meticulously collected and analyzed from the Balochistan livestock population were positively identified as FMDV-positive. This corresponded to a substantial 90% positivity rate, emphasizing the prevalence of the virus within the population of examined cows and buffaloes. The remaining two samples (10%), did not yield any positive results for FMDV. The distinction between positive and negative samples not only confirmed the endemic nature of FMD in the region but also highlighted the significance of comprehensive surveillance and the potential need for more sensitive diagnostic instruments in certain cases (Figure 2). The prevalence of the 'O' serotype of FMD Virus among buffaloes and cows in the provided sample was analyzed and it was found that 33.33 percent of the animals confirmed to have the 'O' serotype were buffaloes, and 66.67% were cows ($p>0.05$). It indicated that the difference in prevalence of 'O' serotype between buffaloes and cows in the sample was not statistically significant (Table 5).

Figure 1

Visual Representation of Cytopathic Effects of LFBK cells infected with FMD virus

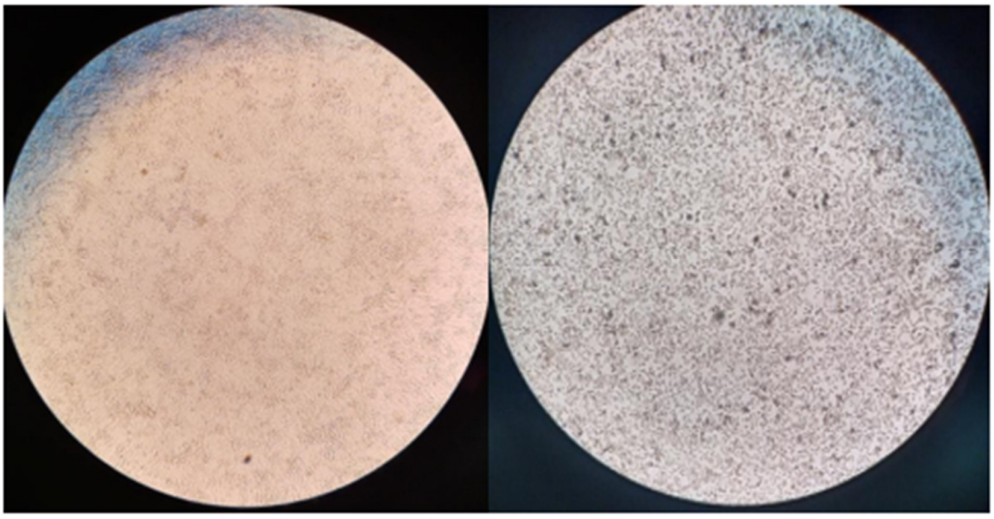

Table 3

Data Collection and Serotyping of FMDV in Cattle and Buffalo from Diverse Balochistan Locations

S. No	Province	Area ofcollection	Sex of animal	Specie(Cattle/Buffalo)	Age (years)	Serotype
PK-01	Balochistan	Quetta	Female	Buffalo	6	O
PK-02	Balochistan	Quetta	Female	Cattle	3	O

S. No	Province	Area ofcollection	Sex of animal	Specie(Cattle/Buffalo)	Age (years)	Serotype
PK-03	Balochistan	KilliMalazaiQuetta	Female	Cattle	4	O
PK-04	Balochistan	KilliAlmasQuetta	Female	Cattle	4	O
PK-05	Balochistan	Jan MuhammadRoadQuetta	Female	Buffalo	5	O
PK-06	Balochistan	KilliAlmo Quetta	Female	Buffalo	5	O
PK-07	Balochistan	Jan Muhammad RoadQuetta	Female	Buffalo	5	O
PK-08	Balochistan	Pishin	Female	Cattle	5	O
PK-09	Balochistan	RasaniRoadQuetta	Female	Cattle	3	O
PK-10	Balochistan	SamaliSalamKilli Quetta	Female	Cattle	4	O
PK-11	Balochistan	Noshki	Female	Cattle	4	O
PK-12	Balochistan	Noshki	Female	Cattle	4	O
PK-13	Balochistan	Jan MuhammadRoadQuetta	Female	Buffalo	4	O
PK-14	Balochistan	Pishin	Female	Cattle	5	O
PK-15	Balochistan	Mastung	Female	Cattle-Calf	1	O
PK-16	Balochistan	Jan MuhammadRoadQuetta	Female	Buffalo	5	O
PK-17	Balochistan	Pishin	Female	Cattle	4	O
PK-18	Balochistan	Pishin	Female	Cattle	4	O

Table 4

CT Values for Detection of FMDV in Samples with Corresponding Lab-IDs

S.NO	LAB-ID	CTVALUES
01	PK-01	------
02	PK-02	------
03	PK-03	------
04	PK-04	19
05	PK-05	18
06	PK-06	18
07	PK-07	37
08	PK-08	-------
09	PK-09	20
10	PK-10	20
11	PK-11	36
12	PK-12	21
13	PK-13	31
14	PK-14	32
15	PK-15	21
16	PK-16	15

Figure 2

Comparison of RT-PCR Gel Electrophoresis Bands for Sample and Positive Control Results

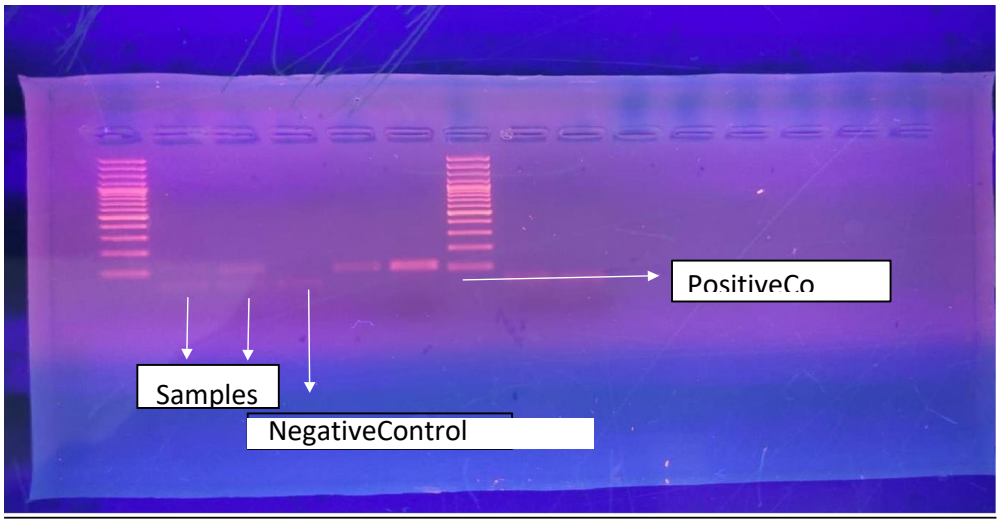

Table 5

Prevalence and Distribution of FMDV 'O' Serotype Among Buffaloes and Cows in Balochistan

S. No	Serotypes	Buffaloes n(%)	Cows n(%)	Total n(%)	χ2	p-value
1	O	6 (33.33)	12 (66.67)	18 (100)	0.7594	0.3835

Discussion

The purpose of this research was to identify and profile the predominant FMDV serotypes in Balochistan cattle and buffalo populations because this disease has a significant economic impact on the cattle. Our findings provided crucial insights into these circulating FMDV genotypes, paving the way for the creation of individualized control strategies. Another study evaluated 590 samples for FMD virus and 180 of these samples tested positive (33.2%). At 20.7%, FMDV 'O' serotype was the leading cause of outbreaks, followed by serotypes A and Asia-1. Notably, there were no 'C' serotype cases. The data revealed a greater prevalence of disease in agroclimatic zones than in upland regions. In Pakistan, the prevalence of FMDV among cattle and buffaloes was 33.2% based on 590 samples representing over 50 outbreaks. Cattle, at 37.1% prevalence, were more prevalent than bison, at 28.0%. In addition, eight instances of co-infections with multiple serotypes were identified, suggesting an endemic disease condition. This analysis, like ours, found the highest prevalence of O serotype within the study population (Abubakar et al., 2012).

Our findings were in agreement with the study conducted on 116 FMDV isolates from outbreaks and persistently infected animals that were characterized molecularly. Sequencing of the VP1 region revealed a close relationship between carrier and field viruses, which could lead to new outbreaks of FMD in cattle closely housed with buffaloes. FMDV serotype A, which was associated with Topotype ASIA, possessed multiple lineages with 23 variable sites in the VP1 region. Serotype O, which was related to the Pan Asia 2 Middle East South Asia topotype, possessed multiple lineages [12]. Similar research was conducted in India in 2021 that sought to determine the genetic lineages of FMDV isolates from outbreaks in 2019-2022. Two primary lineages, O/ME-SA/Ind2001e, and O/ME-SA/Cluster-2018, were found to be responsible for 2021 epidemic-scale outbreaks, challenging the notion that such outbreaks in India are typically associated with a single lineage. Notably, the sub-lineage O/ME-SA/PanAsia-2/ANT10 was identified in India and was linked to outbreaks in Jammu and Kashmir. Both the O/ME-SA/ind2001e and O/ME-SA/Cluster-2018 lineages have expanded geographic distributions and the potential to propagate beyond their current regions. All field isolates were antigenically matched to

the current Indian vaccine strain, indicating the vaccine's effectiveness against these lineages (Dahiya et al., 2023).

The consistent prevalence of FMDV 'O' serotype across all samples was one of the most striking findings. This suggested that the dominant strain (type O) was circulating in the region. Therefore, the prevalence of serotype 'O' in our findings indicated an urgent need to prioritize serotype-specific vaccines in Balochistan in order to better manage FMD. Similar to our study, the investigation of 80 serotype O of FMD viruses originating from East and North Africa was isolated. The individuals were characterized using viral neutralization assays and capsid sequencing techniques. The majority of field isolates exhibited significant serological cross-reactivity with both local and international vaccine strains, indicating that immunization has the potential to be an effective means of controlling serotype O of FMD(Lloyd-Jones et al., 2017)

Moreover, the results of RT-PCR demonstrated the test's sensitivity by identifying an astounding 90% sample positivity rate. This discovery demonstrated the prevalence of FMDV in Balochistan and elevated the reliability of RT-PCR as a diagnostic tool. Nonetheless, our observations of the LFBK cell line revealed several intriguing nuances. CPEs (a hallmark of viral presence) were detected in the majority of samples. This raised concerns regarding the possible existence of strains or viral loads that evaded detection by conventional methods. Alternately, external factors, such as sample handling idiosyncrasies, transportation conditions, or even the initial viral load at the point of collection, could affect the results. The emphasis on meticulous record-keeping in our study, as evidenced by the detailed Performa for each sample, provided a comprehensive epidemiological perspective. The extensive data collected, which included vaccine types, husbandry practices, and clinical symptoms, facilitated more in-depth analysis and disclosed the correlations influencing the spread and manifestation of disease.

Similar to our investigation, another study employed an RT-PCR assay for specific identification and differentiation of FMDV serotypes O, A, and Asia 1 using clinical samples obtained from field settings. A set of specialized primers and probes was developed by formulating them using 571 VP1 coding area sequences that were divided into seven pools. The utilization of multiplex real-time RT-PCR, employing the specified primers and probes, demonstrated superior sensitivity and serotype-specific identification in comparison to conventional VP1 RT-PCR/sequencing techniques when applied to reference FMDVs. When subjected to experimentation using FMDV field viruses that were cultured in cell culture, a complete concordance of serotyping was observed between the novel multiplex real-time RT-PCR and the previous technique. This approach demonstrated a serotyping accuracy of 92.7% for field clinical samples(Lim et al., 2022).

Considering the future, our findings emphasized the need for heightened surveillance and rigorous, targeted vaccination campaigns. An examination of larger-scale studies could determine if the distribution of the 'O' serotype is consistent in neighboring regions, thereby facilitating a coordinated regional response. The path to FMD control and eventual eradication necessitated multilayered approaches whereby our study played a pivotal role in the development of future interventions (Gunasekera et al., 2022).

Conclusion

In this study conducted in Balochistan, the 'O' serotype of FMDV was observed to predominate among cattle and buffalo populations, indicating the imperative need for serotype-specific interventions. With RT-PCR disclosing the 90% positivity rate, such results highlighted the endemic nature of FMDV in this region, and the need for increased surveillance and strategic vaccination campaigns. The presented data argued for the meticulous and adaptable approach to research and intervention strategies to effectively manage and reduce the prevalence of FMD in the region.

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